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#### 4. Introduction

Increased awareness and systematic screening for breast cancer has resulted in early detection of this disease. However, 20 – 30% of node negative breast carcinoma patients will develop recurrent tumors [Weidner, 1995] and given the highly metastatic nature of breast cancer, many of these patients progress to disseminated disease [Lee, 1983]. Although developments in experimental therapies for the treatment of advanced breast cancer is promising, much of the existing treatment for advanced metastatic cancer is palliative. Among the numerous **symptoms of advanced cancer**, pain remains the most significant determinant of **quality of life** [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleland, 1982]. Despite pain being the most feared symptom of advanced cancer, clinical management of cancer pain remains inadequate. Limitation in the clinical management of advanced cancer pain appears multifactorial ranging from the nature of pain itself to the irrational fear of prescribing large quantities of opiates among the treating physicians [Fife et al, 1993; Zenz & Strumpf, 1993]. A novel non-opioid approach to pain management which requires minimal high-technology resources will have wide spread applications in the management of terminal cancer pain and other intractable pain syndromes.

In this proposal, we describe a **novel viral-vector mediated gene-therapeutic approach to pain management**. This notion is based on well documented observations that all vertebrate nervous system including humans posses an endogenous analgesic system. Of the numerous neurotransmitter systems implicated in such an endogenous analgesic system, one of the best described descending analgesic mechanism is the brain stem serotonergic input to the spinal cord. Stimulation of brain stem neurons result in release of serotonin, an amine neurotransmitter, at the spinal cord which modulates the transmission along the pain pathway. The original goal of this project was to examine if enhancement of this endogenous serotonergic analgesic system can provide analgesia. Our approach was to introduce a recombinant adenovirus designed to express one subtype (5HT3) of the serotonin receptor into the subarachnoid space as a means of overexpressing the 5HT3 receptor in spinal neurons. As documented on the last progress report, we concluded that intrathecal Ad(5HT3-sense) administration had little effect on nociceptive threshold in rats. A reexamination of adenoviral access to the spinal cord proper led us to conclude that a 100 nm adenovirus particle was too large to cross into the spinal cord proper even after subarachnoid administration. This led us to propose an alternative approach to gene therapy for pain management through intrathecal injection of antisense oligonucleotide targeting protein kinase C- $\gamma$ , an intracellular signal transduction molecule well documented to play a critical role in neuropathic pain.

#### 5. Body

In this reporting period (Year II of the project), we focused our efforts on the development of an alternative strategy for gene-based therapy for neuropathic pain. Specifically, we targeted an intracellular pronociceptive signal transduction molecule, protein kinase C- $\gamma$ , strongly implicated in mediating the pain cascade [Mao et al, 1995; Malmberg et al, 1997], and began investigating the use of antisense oligonucleotide [Agrawal & Temsamani, 1996; Akhtar & Agrawal, 1997] as a potential therapeutic drug. The antisense oligonucleotide approach was chosen because the PI believes that this strategy, in addition to its proven utility as a selective

drug in other systems, is a technology most likely to lead into a human clinical trial in the near future [Akhtar & Agrawal, 1997; Diasio & Zhang, 1997].

The following progress have been made on the revised Task II stated on the last progress report:

*Task II:*

- *perform further *in vitro* antisense oligonucleotide PKC- $\gamma$  knock down experiments to verify the mechanism of protein knock down.*

1. Several candidate antisense oligonucleotides were identified based on sequence comparison of the classical PKCs (figure 1). *In vitro* antisense knock down experiment demonstrates differential efficacy in reducing the amount of PKC $\gamma$  protein (figure 2). All 11 oligonucleotides tested to date (O1 – O11) demonstrate some degree of reduction in PKC- $\gamma$  protein. O4, O6, and O9 demonstrating best knock down. The result for O6 is in agreement with our preliminary data presented on our last progress report. In contrast, O7 show only a modest efficacy.
2. We have encountered problems in demonstrating efficient antisense knock down of PKC- $\gamma$ , *in vivo*. Various dosing regimen have been tried (up to 100  $\mu$ g / dose x q 12 hours) without consistent correlation between behavioral analgesia and biochemical evidence of spinal cord PKC- $\gamma$  protein reduction. We have revised our behavioral pain assay from a partial sciatic nerve ligation to a formalin (inflammatory) model of pain. Since chronic pain associated with cancer often exhibits a strong inflammatory component, the formalin model may be more appropriate.

During the next funding period, the following aims will be accomplished:

- a. A time course experiment to develop an optimal protocol for *in vivo* knock down using Western blot assay of spinal cord homogenate.
- b. A time course study to examine the return of PKC- $\gamma$  protein level after cessation of the antisense treatment. This information will help design an *in vivo* protocol to examine whether interruption of the pain cascade will provide a long-term relief from pain. Alternatively, pain could return immediately after cessation of the antisense treatment as the cells regenerate PKC- $\gamma$ .
- c. *In vivo* studies examining anti-nociceptive effects of anti-PKC- $\gamma$  antisense oligonucleotide will be continued with focus on the formalin model of inflammatory pain.

## **6. Key Research Accomplishments:**

- Developed an alternative strategy based on antisense oligonucleotide knock down of protein kinase C- $\gamma$  playing a critical role in the neuropathic cascade.
- Screened antisense oligonucleotides to identify a sequence leading to selective PKC- $\gamma$  protein knock down.
- Demonstrated anti-nociceptive effect of intrathecal administration of antisense oligonucleotide targeting PKC- $\gamma$ .

## **7. Reportable Outcomes:**

### Manuscripts / abstracts / presentations:

Wu C, Garry M, Zollo R, Yang J "Gene therapy for the management of pain Part I: Methods and strategies", Anesthesiology, (in revision).

Wu C, Garry M, Zollo R, Yang J "Gene therapy for the management of pain Part II: Molecular targets", Anesthesiology, (in revision).

Zollo R, Malik S, Yu J, Yang J "Antisense oligonucleotide-mediated selective knock down of protein kinase C  $\gamma$ ", (in preparation).

Yang J, Garry M, Zollo RA "Protein kinase C-gamma and neuropathic pain", Abstract FF-11, Era of Hope Meeting, 6/8-12,2000, Atlanta, GA.

## **8. Conclusions:**

Inadequate management of cancer pain has been widely documented and may effect a reduction of quality of life in terminal patients [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleeland, 1982]. Furthermore, neuropathic pain, which may be present with advanced cancer is generally resistant to opioid therapy [Payne, 1993; Arner & Myerson, 1988]. Development of a non-opiate, non-addictive, long-lasting therapy for cancer pain will revolutionize clinical cancer pain management and alleviate patient suffering. The goal of this proposal is to investigate the feasibility of a gene-therapeutic approach to pain management. Should this approach prove feasible, in future work, this concept can be extended to overexpression of other neurotransmitter receptors implicated in the antinociceptive action (e.g. GABA<sub>A</sub>, adrenergic  $\alpha$ 2, etc.) or underexpression of receptors mediating the nociceptive actions (e.g. NMDA, tachykinin, etc.) through antisense knock down.

The original goal was to use the recombinant adenovirus as a vehicle for overexpressing antinociceptive serotonin type 3A receptors in the spinal cord as a means of providing pain relief. Our results over the first 12 months indicated that the recombinant adenovirus delivered by the subarachnoid route will not work. The physical barrier probably due to the marginal glial cells of the spinal cord prevents the adenovirus from transducing spinal cord neurons. Without efficient transduction of the spinal cord proper, the proposed approach will not work. Direct injection of the virus into the spinal cord is an alternative delivery method, but remains unsatisfactory because the approach is too invasive for easy translation to the human clinical arena.

We have taken an alternative strategy to accomplish the same goal of developing a treatment for neuropathic pain. During the present reporting period, we explored antisense oligonucleotide targeting spinal cord protein kinase C- $\gamma$  as a therapeutic strategy for a non-opioid pain management. Progress thus far has identified several candidate antisense oligonucleotides that exhibit PKC- $\gamma$  protein knock down. We will continue to this investigate antisense oligonucleotide as a novel alternative to opioids for treating neuropathic pain. Antisense oligonucleotide is now well accepted as a form of therapy in humans. It is our goal to thoroughly investigate the antisense oligonucleotide

targeting spinal cord PKC- $\gamma$  in a preclinical model and move towards implementing a clinical trial in humans. Task for year 3 of funding will focus on in vivo assay of efficacy of PKC- $\gamma$  antisense oligonucleotide. We hope to move a step closer to the goal of developing a novel and effective pain relief with reduced side-effects for combating the diverse types of pain associated with advanced cancer. In this respect, the antisense oligonucleotide approach may be more practical than the viral vector approach.

## 9. References:

Agrawal S, Temsamani J (1996) *Comparative pharmacokinetics of antisense oligonucleotides*, In: Methods in Molecular Medicine- Antisense Therapeutics (ed. S. Agrawal), Humana Press, NJ, pp 247 – 270).

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Fife, B.L., Irick, N. and Painter, J.D. (1993) *A comparative study of the attitudes of physicians and nurses toward the management of cancer pain*. J. Pain Symptom. Management **8**: 132 - 139.

Heim, H.M. and Oci, T.P.S. (1993) *Comparison of prostatic cancer patients with and without pain*. Pain **53**: 159 - 162.

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Malmberg A, Chen C, Tonegawa S, Basbaum AI (1997) *Preserved acute pain and reduced neuropathic pain in mice lacking PKC- $\gamma$* , Science **278**: 279 – 283.

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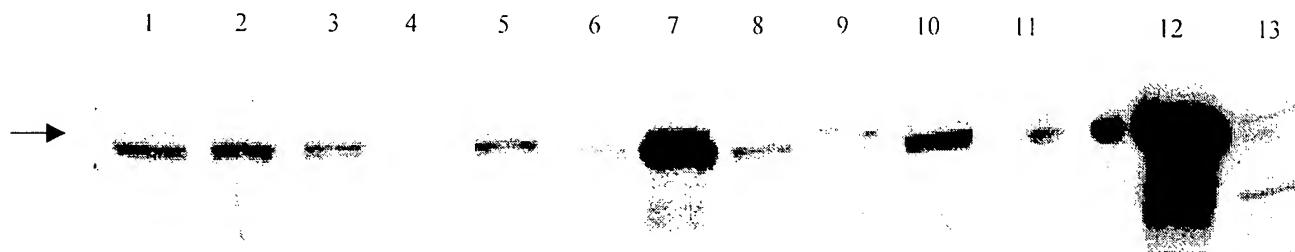
Pourtenoy, R.K. (1990) *Pain and quality of life: clinical issues and implications for research*. Oncology **4**: 172 - 180.

Weidner N (1995) *Prognostic factors in breast carcinoma*, Current Opin. Ob & Gyn **7**: 4 – 9.

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**Figure 1: Amino acid sequence alignment of classical rat PKC isoenzymes.** The entire coding sequence for PKC- $\alpha$  (E04372), PKC- $\beta$ 1 (M19007), and PKC- $\gamma$  (E04371) were aligned using DNAsis (Hitachi Inc, CA). Genebank accession numbers are given in parenthesis. Regions of significant amino acid divergence were identified and thirteen oligonucleotides corresponding to the selected amino acid regions were defined as the candidate antisense oligonucleotides noted as O1 – O13 (noted in red). The PS oligonucleotide (in blue) was designed against a conserved region in the pseudosubstrate domain. Three of the well defined functional domains of the classical PKCs are identified by the green brackets. PKC- $\beta$ II with 95% amino acid identity with  $\beta$ I was omitted from the figure for clarity.

	O1	O2	PS																																			
1	<b>MADWYV</b> PANDS	TASQDWANRF	ARKGALRQKN	WHEVKDHKFI	ARFFKQPTFC																																	
1	MADPAA	PPP	SEGEESTVRF	ARKGALRQKN	WHEVKNHKFI	ARFFKQPTFC																																
1	MAGLGPGGGD		SEEGPRPL-F	CRKGALRQKV	WHEVKSHKFT	ARFFKQPTFC																																
..	60	70	80	90	100																																	
51	SHCTDFI	WGF	GKQGFQ	CQVC	CFVWHR	RCHE	FWTFS	CPGAD	KGPD	TDDP	RS																											
51	SHCTDFI	WGF	GKQGFQ	CQVC	CFVWHR	RCHE	FWTFS	CPGAD	KGPAS	DDP	RS																											
51	SHCTDFI	WGF	GKQGL	QCQVC	SFWVH	RRRCHE	FWTFE	CPGAG	KGPQT	DDP	RS																											
	110	120	130	140	150																																	
101	KHKFKI	HTY	G	SPTFC	DHC	GS	LLYGLI	HQGM	KCDTC	DMNWH	KQC	VIN	VPSL																									
101	KHKFKI	HTY	S	SPTFC	DHC	GS	LLYGLI	HQGM	KCDTC	DMNWH	KRC	VUN	VPSL																									
101	KHKFR	LEH	SYS	SPTFC	DHC	GS	LLYGLV	HQGM	KSCC	DMNWH	RRC	VRS	VPSL																									
	160	170	180	190	200																																	
151	CGMDH	TEKE	RG	RIVL	KA	EY-D	D	DEK	LH	TYW	RD	A	WNL	IPMDP	N	GLSDP	YV	VKLE																				
151	CGTDH	TER	RG	RIVI	QAH	I-D	R	EV	LI	WW	RD	A	WNL	VPM	DP	N	GLSDP	YV	VKLE																			
151	CGVDH	TER	RG	RLO	LEI	RAP	T	SDE	I	HIT	WGE	A	WNL	IPM	DP	N	GLSDP	YV	VKLE																			
	210	220	230	240	250																																	
201	LIPDP	PKWESK	QKTKT	IR	STL	W	F	W	Q	F	W	K	L	KP	SD	KD	R	LSVE	I	WDW	PF																	
201	LIPDP	PKSESK	QKTKT	IK	CSL	W	F	W	Q	F	W	K	L	KP	SD	KD	R	LSVE	I	WDW	DL																	
201	LIPDP	PKWLT	QKTKT	V	KATL	W	F	W	Q	F	W	K	L	KP	GD	W	ERR	LSVE	V	WDW	DF																	
	260	270	280	290	300																																	
251	TSRND	FMGSL	SFGV	SEL	M	W	P	W	Q	E	Y	T	W	P	E	G	D	E	C	W																		
251	TSRND	FMGSL	SFG	I	SEL	Q	K	A	C	W	G	F	K	L	S	E	E	G	E	N	W																	
251	TSRND	FMGAM	SFGV	SEL	L	K	W	P	W	Q	E	T	Y	K	L	S	E	G	E	N	W																	
	310	320	330	340	350																																	
301	ELRQK	FE	---	---	KAK	L	G	F	A	G	N	K	V	I	S	E	D	---	P	---	S	M	I	E	V													
301	ELRQK	FE	---	---	RAK	I	Q	O	G	T	K	A	P	E	I	T	A	N	T	I	S	K	F	D	M													
301	SLLQK	FEACN	Y	P	LELY	E	V	R	M	G	F	S	S	P	I	S	P	T	D	S	E	C	F	G	A	S	P	G	E									
	360	370	380	390	400																																	
351	KLTDF	NFLMV	LGKGS	FGK	W	L	A	D	E	K	G	T	E	E	L	Y	A	I	K	L	K	D	W	Y	Q	DD	D	W	E	C								
351	KLTDF	NFLMV	LGKGS	FGK	W	L	S	E	R	K	G	T	D	E	L	Y	A	I	K	L	K	D	W	Y	Q	DD	D	W	E	C								
351	HISDR	FSFLMV	LGKGS	FGK	W	L	A	E	R	R	G	S	D	E	L	Y	A	I	K	L	K	D	W	Y	Q	DD	D	W	C									
	410	420	430	440	450																																	
401	MWEK	RVLALL	D	X	---	PPF	L	T	Q	L	H	S	C	Q	T	V	D	R	L	Y	F	M	E	Y	W	NGG	D	L	M	Y	H							
401	MWEK	RVLALP	G	K	---	PPF	L	T	Q	L	H	S	C	Q	T	V	D	R	L	Y	F	M	E	Y	W	NGG	D	L	M	Y	H							
401	LWEK	RVLALG	E	R	G	P	G	R	P	H	F	T	L	H	S	T	Q	D	R	L	Y	F	M	E	Y	W	NGG	D	L	M	Y	H						
	460	470	480	490	500																																	
451	QQVG	KFKE	PQ	A	V	F	Y	A	E	I	S	I	G	L	F	L	H	K	R	G	I	Y	R	D	E	G	H	I	K	I								
451	QQVG	RFKE	PH	A	V	F	Y	A	E	I	A	I	G	L	F	L	Q	S	K	G	I	Y	R	D	E	G	H	I	K	I								
451	QQLG	KFKE	PH	A	A	V	F	A	E	I	A	I	G	L	F	L	H	N	Q	G	I	Y	R	D	E	G	H	I	K	I								
	510	520	530	540	550																																	
501	ADFGM	CKEHM	MDG	V	T	RT	F	C	G	D	P	D	Y	I	A	P	E	I	A	Y	Q	P	Y	G	K	W	L	Y	Q	DD	D	W	C					
501	ADFGM	CKEHM	MDG	V	T	RT	F	C	G	D	P	D	Y	I	A	P	E	I	A	Y	Q	P	Y	G	K	W	L	Y	Q	DD	D	W	C					
501	TDFGM	CKEHN	V	F	P	E	S	T	R	T	F	C	G	D	P	D	Y	I	A	Y	Q	P	Y	G	K	W	L	Y	Q	DD	D	W	C					
	560	570	580	590	600																																	
551	EMLAG	QPPFD	G	E	D	E	D	L	F	Q	S	I	M	E	H	N	V	Y	P	K	S	L	S	K	E	A	V	S	I	K	G	L	M	T	K	H	P	A
551	EMLAG	QAPFD	G	E	D	E	D	L	F	Q	S	I	M	E	H	N	W	Y	P	K	S	L	S	K	E	A	V	S	I	K	G	L	M	T	K	H	P	G
551	EMLAG	QPPFD	G	E	D	E	D	L	F	Q	S	I	M	E	Q	T	V	Y	P	K	S	L	S	K	E	A	V	S	I	K	G	L	M	T	K	H	P	G
	610	620	630	640	650																																	
601	RLGCG	FEGER	D	V	R	E	H	A	F	F	R	R	I	D	W	E	K	W	R	I	Q	P	F	K	P	C	G	K	A	E	M	F	D	K				
601	RLGCG	FEGER	D	I	K	E	H	A	F	F	R	R	I	D	W	E	K	W	R	I	Q	P	F	K	P	C	G	K	A	E	M	F	D	K				
601	RLGCG	FEGER	D	T	I	R	A	H	G	F	F	R	R	I	D	W	E	K	W	R	I	Q	P	F	R	P	C	G	R	S	E	M	F	D	K			
	660	670	680	690	700																																	
651	FFTR	GQFVLT	P	P	D	Q	L	V	I	A	N	I	D	Q	S	D	F	E	G	F	S	V	W	P	Q	F	V	H	I	L	Q	A	-----					
651	FFTR	QPVELT	P	T	D	K	L	F	I	M	N	D	Q	N	E	F	A	G	F	S	T	T	N	P	F	V	W	-----	I	N								
651	FFTR	AAFAALT	P	P	D	R	L	V	L	A	S	I	D	Q	A	D	F	O	G	T	W	V	N	P	D	F	V	H	D	A	R	P	T	S	P	V	P	V
	710	720	730	740	750																																	
701	W*	.....	.....	.....	.....																																	
701	W*	.....	.....	.....	.....																																	
701	VM*	.....	.....	.....	.....																																	



**Figure 2: Differential reduction of PKC- $\gamma$  by selected antisense oligonucleotides.** Western blot of PKC- $\gamma$  protein in antisense oligonucleotide-treated cells. Lanes 1 – 11 corresponds to antisense oligonucleotides O1 – O11 shown in figure 1. Lane 12 is a positive control without oligonucleotide treatment and lane 13 is a negative control. Individual 35 mm tissue culture dishes 70% confluent with HEK293 cells were co-transfected with PKC $\gamma$ -pCIneo (0.2  $\mu$ g / dish) and 1.0  $\mu$ g / dish oligonucleotide using Lipofectamine Plus (Gibco). Twenty four hours after termination of transfection, cells were harvested and equal amount of protein loaded and separated by SDS gel electrophoresis.